Changes in intracellular free calcium levels of cultured human epidermal keratinocytes exposed to sulfur mustard

Marijke A.E. Mol

TNO Medical Biological Laboratory,
P.O. Box 5815, 2280 HV Rijswijk,
The Netherlands

ABSTRACT

Evidence is growing that sulfur mustard not only causes DNA damage, but also acts by other toxic mechanisms. Increase in the intracellular cytosolic free Ca^{++} ($[Ca]_i$) levels might be one of the pathways leading to cytotoxicity. The $[Ca]_i$ in monolayers of human epidermal keratinocytes exposed to sulfur mustard has been measured by means of the fluorescent indicator Fura-2. A ratio approach was used by monitoring the fluorescent intensity of Fura-2 at excitation wavelengths of 340 and 359 nm with an emission wavelength of 510 nm. So far, preliminary results have been obtained, which indicate that exposure to 50, 100 or 200 µM of sulfur mustard causes little or no changes in $[Ca]_i$ of cultured human epidermal keratinocytes during the first 7 hr after treatment.

This work was supported by US Army Medical Research and Development Command Grant Order DAMD 17-92-V-2008.

COMPONENT PART NOTICE

THIS PAPER IS A COMPONENT PART OF THE FOLLOWING COMPILATION REPORT:

TITLE: Proceedings of the Medical Defense Bioscience Review (1993)
Held in Baltimore, Maryland on 10-13 May 1993. Volume 1.

TO ORDER THE COMPLETE COMPILATION REPORT, USE AD-A275 667.

THE COMPONENT PART IS PROVIDED HERE TO ALLOW USERS ACCESS TO INDIVIDUALLY AUTHORED SECTIONS OF PROCEEDING, ANNALS, SYMPOSIA, ETC. HOWEVER, THE COMPONENT SHOULD BE CONSIDERED WITHIN THE CONTEXT OF THE OVERALL COMPILATION REPORT AND NOT AS A STAND-ALONE TECHNICAL REPORT.

THE FOLLOWING COMPONENT PART NUMBERS COMPRISE THE COMPILATION REPORT:

AD#: P008 752 thru P008 794 AD#: _____

AD#: _____ AD#: _____

AD#: _____ AD#: _____

Accesion For	
NTIS CRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

DTIC FORM 463 OPI: DTIC-TID

INTRODUCTION

Ca^{++} may play a vital role in the onset of toxicological processes leading to cell death (1-3). Disruption of the intracellular Ca^{++} homeostasis may cause an increase of the intracellular cytosolic free Ca^{++} ($[Ca]_i$) levels. Possibly, this will initiate changes in the cytoskeleton as well as degradative cell processes, including activation of certain proteases and nucleases. As these mechanisms might be involved in the pathology of blister formation due to sulfur mustard exposure, it has been investigated whether deregulation of Ca^{++} homeostasis is involved in sulfur mustard-induced toxicity in cultured human epidermal keratinocytes (HEK).

METHODS

Materials

Fura-2 free acid and Fura-2 acetoxymethyl ester (Fura-2 AM) were purchased from Molecular Probes (Eugene, OR); Pluronic F-127 from Calbiochem (San Diego, CA); digitonin, ionomycin, $CaCl_2$, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and cell culture supplies from Sigma (St. Louis, MO).

Cell culture method

Secondary cultures of HEK were grown on glass coverslips that were covered with a feeder layer of irradiated 3T3 mouse fibroblasts. The culture medium was a mixture of DMEM and Ham's F12 medium (3:1), supplemented with 10% bovine calf serum, 10^{-10} M cholera toxin, 0.4 µg/ml hydrocortisone and 10 ng/ml epidermal growth factor and 50 µg/ml gentamicin (4). At day 7 after plating the remnants of the feeder layer were removed by vigorously pipetting. The HEK were then fed with Clonetics Keratinocyte Growth Medium (KGM), a serum-free culture medium with a low Ca^{++} concentration of 0.15 mM. At day 10, when the HEK had nearly reached confluence, the monolayers were used for experiments.

Loading

The method of Tang and Ziboh was used (5). In brief, cells were incubated at RT during 60 min with 5 µM Fura-2 AM in a HEPES buffered saline solution (pH 7.3),

containing in mM: NaCl 110; $MgSO_4$ 1; KCl 5.4; KH_2PO_4 0.44; NaH_2PO_4 0.35; glucose 5; glutamine 2; Hepes 25; $CaCl_2$ 0.15. To facilitate dye uptake, 0.03% Pluronic F-127 was added. Unincorporated fluorescent dye was removed by washing the HEK with buffer. Subsequently, the cells were held for another 60 min in buffer at RT to allow intracellular deesterification.

Assessment of intracellular deesterification

To assess whether the ester has been hydrolyzed by intracellular hydrolases, Fura-2-loaded cells were incubated with 0.01% digitonin in HEPES buffer for 20 min at RT. This treatment is suffient to release all cytosolic Fura-2. Then excitation spectra of the cell-bound Fura-2 were recorded in the presence of low Ca^{++} concentration (EGTA) and high Ca^{++} concentration ($CaCl_2$). These spectra were compared to excitation spectra of Fura-2 free acid in the same buffer.

Ca^{2+} measurement in HEK monolayers on glass coverslips

Measurements were performed in a temperature-controlled cuvette (37°C), housed in a spectrofluorimeter (SLM-Aminco 500C). Changes in fluorescence were monitored at excitation wavelengths of 340 and 359 nm by manual adjustment of the wavelengths. The emission wavelength was 510 nm.

Coverslips were positioned diagonally in the cuvette, containing HEPES buffer.

$[Ca]_i$ were estimated from ratio measurements of Fura-2 fluorescence (340/359 nm excitation) using the following equation (Eq.1):

$$[Ca]_i = K_d[(R - R_{min})/(R_{max}-R)](S_{f2}/S_{b2})$$

K_d is the dissociation constant (220 nM at 37°C). R is the ratio of the sample, R_{min} and R_{max} are the ratios obtained with cells containing very low and very high Ca^{++} levels, respectively. The parameter S_{f2}/S_{b2} is the ratio of fluorescence values measured at 359 nm before/after $CaCl_2$ addition.

To determine R_{min}, cells were loaded with Fura-2 in a Ca^{++} free buffer containing 1 mM EGTA and the ratio of fluorescence intensity at the excitation wavelength pair 340/359 nm was recorded. To provide R_{max}, $[Ca]_i$ of the keratinocytes was raised by enhancing the Ca^{++} concentration in the cuvette to 0.5 mM, together with the addition of ionomycin (final concentration 10 µM). The following values for R_{min} and R_{max} were obtained: 0.75 and 1.32, respectively.

Sulfur mustard exposure

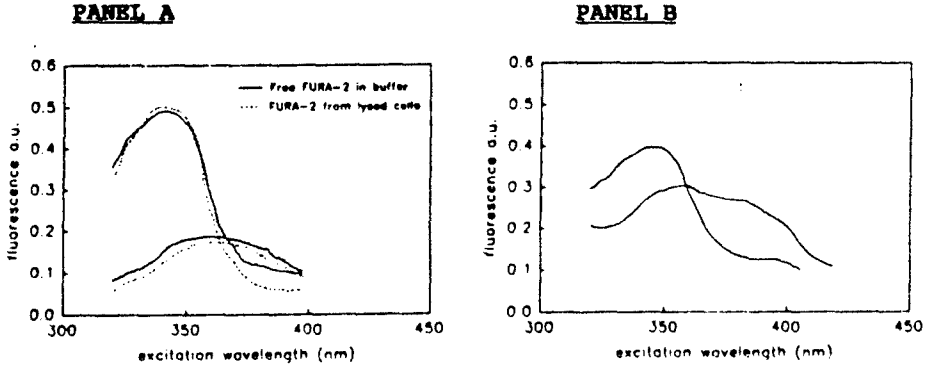
HEK were incubated for 30 min with various concentrations of sulfur mustard in KGM at RT. Cells were loaded with dye after exposure, except for the case that $[Ca]_i$ was measured 1 hr after sulfur mustard treatment.

R E S U L T S

Fura-2 fluorescence spectra

Fura-2 has been loaded into the HEK as its acetoxymethyl ester (Fura-2AM). Cytoplasmic esterases cleaved the acetoxymethyl ester bonds, yielding free Fura-2 into the cytosol. The fluoroscence spectra of Fura-2 released from cells lysed by 0.01% digitonin (Fig 1A, dashed lines) were nearly identical to those of Fura-2 free acid in the same buffer (Fig 1A, solid lines). This indicates the absence of unhydrolyzed Fura-2AM in the cell lysate. These spectra show that non-Ca^{++} bound Fura-2 fluoresces maximally at 380 nm, whereas the fluorescent maximum switches to 340 nm when Ca^{++} is bound to Fura-2. This wavelength pair is generally used for ratio measurements of $[Ca]_i$ (6).

Figure 1. Excitation spectra of Fura-2 free acid in HEPES buffer (panel A) and of Fura-2 released from HEK loaded with Fura-2AM (panel B).



However, the excitation spectrum of non-Ca^{++} bound free Fura-2 in situ in HEK in monolayer on glass coverslips showed a definite shoulder at 390 nm (Fig 1B), which probably is the excitation peak of Fura-2 AM (7). The presence of this shoulder interferes with the use of 380 nm as the second excitation wavelength for ratio measurements. Therefore, the wavelength pair of 340/359 nm has been used for ratio estimation instead of the 340/380 nm pair.

$[Ca]_i$ of HEK exposed to sulfur mustard.

When HEK were exposed during 30 min to 50, 100 or 200 µM of sulfur mustard this resulted in little or no changes in $[Ca]_i$ at 1, 4 or 7 hr after exposure compared to the $[Ca]_i$ of untreated controls at these time points (Table 1). Two small changes were observed. One is a slight decrease of $[Ca]_i$ at 1 hr after exposure to 100 µM sulfur mustard. The other is a little increase in cells that were exposed to 50 µM at 7 hr after exposure.

TABLE 1. $[Ca]_i$ (nM) of HEK exposed to sulfur mustard (x ± s.d.; n = 6 for each point).

Conc. HD (µM)	Time after exp. (hr) 1	4	7
0	148±10	101±7	128±6
50			154±13
100	110±8	110±8	138±10
200	138±7	101±4	

D I S C U S S I O N

For a meaningful interpretation of the presented data, it should be kept in mind that they were based on a limited number of experiments and were obtained under suboptimal experimental conditions since the available spectrofluorometer was not quite fitted for $[Ca]_i$ measurement with Fura-2. As a stirring device in the cuvette was lacking, cells in suspension could not be used. Therefore, cells were grown on coverslips which were placed diagonally in the cuvette. It appeared that after loading, unhydrolyzed Fura-2 AM remains attached to the cells. Probably, the ester is bound to cell membranes or trapped in organelles, both sites at which it cannot be reached by esterases. According to Malgaroli et al. (7), who reported the same observations, the isosbestic point, 359 nm, was used as the second wavelength for ratio estimation. Although the fluorometer was not equipped for continuous dual excitation wavelength measurements, ratio estimation obtained by manual adjustment of the wavelengths was preferred over single wavelength measurements, since a number of variables that could perturb the measurements are eliminated by using a fluorescence ratio.

Considering the thus obtained data, it has been concluded that, although some small changes in the $[Ca]_i$ of HEK were observed, exposure of HEK to concentrations of sulfur mustard up to 200 µM does not lead to an elevation of $[Ca]_i$ levels within 7 hr after exposure. If exposure to sulfur musturd will cause any raise in $[Ca]_i$, it will be such small that it could not be detected under the present experimental conditions. However, the presented data include neither the assessment of $[Ca]_i$ at very early time points after exposure, nor time periods between 7 and 24 hr. Therefore, an early transient elevation of $[Ca]_i$, or an irreversible increase starting later than 7 hr post-exposure can not be excluded.

REFERENCES

1. Nicotera, P., Bellomo, G., and Orrenius S. (1990) The role of Ca^{2+} in cell killing. Chem. Res. Toxicol. 3, 484-494.
2. Reed, D.J. (1990) Review of the current status of calcium and thiols in cellular injury. Chem. Res. Toxicol. 3, 495-502.
3. Farber, J.L. (1990) The role of calcium in lethal cell injury. Chem. Res. Toxicol. 3, 503-508.

4. Mol, M.A.E., van de Ruit, A.M.B.C., and Kluivers, A.W. (1989) NAD^+ levels and glucose uptake of cultured human epidermal cells exposed to sulfur mustard. Tox. Appl. Pharmacol. 98, 159-165.
5. Tang, W., and Ziboh, V.A. (1991) Agonist/inositol triphosphate-induced release of calcium from murine keratinocytes: a possible link with keratinocyte differentiation. J. Invest. Dermatol. 96, 134-138.
6. Tsien, R.Y., Rink, T.J. and Poenie, M. (1985) Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. Cell Calcium 6, 145-157.
7. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) J. Cell Biol. 105, 2145-2155.